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ABSTRACT

Patients undergoing hypothermic cardiopulmonary bypass are often on aspirin therapy. Hypothermia, aspirin, and cardiopulmonary bypass can each induce a platelet function defect, but it is not known if the effects of aspirin and hypothermia are additive in this regard. To address this question in humans *in vivo*, the forearm skin temperature of healthy volunteers was equilibrated and maintained at either normothermia (32°C) or hypothermia (28°C or 22°C) before and 16 hours after the ingestion of aspirin 650 mg. Hypothermia was achieved by a stream of air cooled by dry ice or placing the volunteer in a 4°C walk-in cold room. Skin temperature was monitored by a surface thermometer. A standardized template bleeding time was performed on the forearm and the shed blood emerging from the wound was assayed for a) platelet surface P-selectin expression by whole blood flow cytometry (reflecting α granule secretion), b) total shed blood hemoglobin and shed blood volume (reflecting blood loss), and c) thromboxane B_2 (the stable metabolite of thromboxane A_2) by radioimmunoassay. Hypothermia resulted in marked prolongation of the bleeding time. Aspirin resulted in prolongation of the bleeding time under normothermic conditions, but did not augment the hypothermia-induced prolongation of the bleeding time. Platelet surface P-selectin up-regulation in shed blood was abolished by hypothermia. Aspirin had no effect on maximal platelet surface P-selectin expression under normothermic or hypothermic conditions. Aspirin did not augment hypothermia-induced blood loss, as determined by total shed blood hemoglobin and shed blood volume. Both hypothermia and aspirin resulted in markedly reduced shed blood thromboxane B_2. Although aspirin slightly augmented the hypothermia-induced reduction in shed blood thromboxane B_2, the concentration of thromboxane generated in shed blood under hypothermic conditions in the absence of aspirin had no effect on platelet surface P-selectin or platelet aggregation in whole blood. In summary, as determined by four independent parameters in the shed blood emerging from a standardized bleeding time wound (blood loss, bleeding time, platelet surface P-selectin, and thromboxane B_2), aspirin does not augment hypothermia-induced platelet dysfunction *in vivo*.

INTRODUCTION

Patients undergoing hypothermic cardiopulmonary bypass are often on aspirin therapy. Hypothermia (1-4), aspirin (5), and cardiopulmonary bypass (6-9) can each induce a platelet function defect, but it is not known if the effects of aspirin and hypothermia are additive in this regard. Therefore, the goal of the present study was to determine whether aspirin augments hypothermia-induced platelet dysfunction *in vivo*. To achieve this goal, we used our previously described human forearm model (4) to study the effects of hypothermia and aspirin on *in vivo* platelet function in the absence of cardiopulmonary bypass.

METHODS

Monoclonal Antibodies

S12 (Centocor, Malvern, PA) is directed against P-selectin (10,11). P-selectin (also referred to as CD62P (12) and previously referred to as GMP-140 and PADGEM protein) is a component of the α granule membrane of resting platelets that is only expressed on the platelet plasma membrane after platelet secretion (10,13). Y2/51 (DAKO, Carpinteria, CA) is directed against platelet membrane GPIIIa (14). S12 was biotinylated, and Y2/51 was conjugated with fluorescein isothiocyanate (FITC), as previously described (15).

Flow Cytometric Analysis of Platelets in Whole Blood Emerging from a Bleeding Time Wound

The flow cytometric method of analysis of platelets in whole blood emerging from a bleeding time wound has been previously described in detail (4,15). The experimental protocol was approved by the Institutional Review Board of the Boston University School of Medicine. Healthy adult volunteers who had not ingested aspirin or other drugs within the previous 10 days participated in the study after providing written informed consent. Peripheral blood samples and bleeding time samples were collected before and 16 hours after the ingestion of 650 mg aspirin.

The forearm skin temperature of the volunteers was equilibrated to either 32°C, 28°C, or 22°C by the following methods. 32°C (normothermia): ambient room temperature. 28°C: application to the forearm of wet ice in a plastic overwrap until the desired temperature was achieved. The temperature was then maintained by a stream of vapor produced by a combination of dry ice and water passed through a desiccator to reduce the moisture. 22°C: application to the forearm of wet ice in a plastic overwrap until the desired temperature was achieved. The volunteer was then placed in a 4°C walk-in cold room. If the temperature of the forearm dropped below 22°C, a hair dryer was used to warm the area. Local skin temperature was monitored by a surface thermometer (Skin Temperature Sensor, Mon-A-Therm, St. Louis, MO) placed within a few millimeters of the bleeding time incision. The bleeding time was not performed until the skin temperature was stable at the desired level. The skin temperature was then monitored throughout the bleeding time measurement procedure and was kept constant.

Standardized bleeding times were performed in duplicate with a Simplate II device (General Diagnostics, Durham, NC) as previously described (16). The blood emerging from the bleeding time wound (shed blood) was collected with a micropipet at 2 minute intervals until the bleeding stopped. After each pipetting, any residual blood at the bleeding time wound site was removed with filter paper. Immediately after collection at each time point, 9 μL of the pipetted blood was added to a microfuge tube containing sodium citrate (resulting in a 1:10 anticoagulant/blood ratio), fixed for 30 minutes at 22°C in 1% formaldehyde (final concentration), and diluted 1:10 by volume in modified Tyrode's buffer.

As previously described,(4,15) the fixed diluted whole blood samples were then incubated (15 minutes, 22°C) with a saturating concentration of biotinylated monoclonal antibody S12 (P-selectin-specific) and a near saturating concentration of FITC-conjugated monoclonal antibody Y2/51 (GPIIIa-specific), incubated (15 minutes, 22°C) with phycoerythrin-streptavidin (Jackson ImmunoResearch, West Grove, PA), and analyzed in an EPICS Profile flow cytometer (Coulter Cytometry, Miami, FL). After identification of platelets by gating on both FITC positivity and their characteristic light scatter, binding of the biotinylated monoclonal antibody was determined by analyzing at least 1,000 individual platelets for phycoerythrin mean fluorescence. Background binding, obtained from parallel samples run with FITC-Y2/51 and biotinylated mouse IgG (Boehringer Mannheim, Indianapolis, IN), was subtracted from each sample. In some experiments, as indicated, platelet surface P-selectin was determined by whole blood flow cytometry in peripheral blood rather than shed blood.

Radioimmunoassay of Shed Blood Thromboxane B_2

After the skin temperature of the forearm of a normal volunteer was equilibrated to either 22°C, 28°C, or 32°C (as described above), all blood emerging from a Simplate II bleeding time wound was collected into heparin 1,000 U/mL and, to halt the production of thromboxane, ibuprofen 1.9 mg/mL (Upjohn, Kalamazoo, MI), as previously described (1,4). The samples were immediately placed on ice and centrifuged (1,650 g, 10 minutes, 4°C). The supernatants were stored at -80°C until the concentration of thromboxane B_2 (a stable metabolite of thromboxane A_2) was assayed with an RIA kit (New England Nuclear, Boston, MA), as previously described (1).

Measurement of Blood Loss from the Bleeding Time Wound

Blood emerging from the bleeding time wound (shed blood) was collected onto Whatman #4 filter paper (Whatman, Maidstone, U.K.). Two methods were used to quantitate the blood loss. Firstly, as previously described (17), after elution by cyanmethemoglobin, total shed blood hemoglobin was measured. Secondly, the volume of shed blood was measured by converting the opaque area on the filter paper into digitized units by means of an NEC computer, a Hewlett-Packard Scanjet scanner, and Sigmascan® digitization software. Control experiments using EDTA anticoagulated whole blood (Becton Dickinson Vacutainer) blotted from a plastic surface in a manner mimicking shed blood collection from a bleeding time wound established that each 100 μL of whole blood produced a 6.2 cm^2 opaque area of the filter paper in a linear fashion from 25 to 400 μL ($n = 49$, $r = 0.989$, $p < 0.001$). Additional control experiments validated that this equation was independent of hematocrit values in the range of 20 - 60 ($n = 20$).

Whole Blood Platelet Aggregometry

After discarding the first 2 mL, peripheral blood from non-aspirinated normal volunteers was drawn into a sodium citrate Vacutainer (Becton Dickinson, Rutherford, NJ) and diluted with an equal volume of 0.9% NaCl. One mL aliquots were placed in an impedance method whole blood aggregometer (Chronolog 500Ca, Havertown, PA) interfaced with a Pentium PC computer. The samples were warmed to 37°C and stirred at 1,000 rpm. Ten µL of a 100-fold concentration of the thromboxane A_2 analogue U46619 (Cayman Chemical, Ann Arbor, MI) diluted in normal saline was added, and the amplitude (ohms) of platelet aggregation was recorded for 5 minutes. All aggregometry experiments were completed within 90 minutes of blood collection.

RESULTS

Prior to the ingestion of aspirin, hypothermia resulted in marked prolongation of the bleeding time (Fig. 1). Ingestion of aspirin resulted in prolongation of the bleeding time under normothermic conditions, but did not augment the hypothermia-induced prolongation of the bleeding time (Fig. 1).

The up-regulation of the platelet surface expression of P-selectin in the shed blood emerging from the bleeding time wound was abolished by hypothermia (Fig. 2, left panel). Aspirin had no significant effect on maximal platelet surface P-selectin expression in shed blood under normothermic or hypothermic conditions (Fig. 2).

Blood loss from the bleeding time wound was determined by total shed blood hemoglobin (Fig. 3A) and by shed blood volume (Fig. 3B). Although aspirin resulted in increased blood loss at normothermic temperatures, aspirin did not augment hypothermia-induced blood loss (Figs. 3A and 3B).

Thromboxane B_2 in shed blood was markedly reduced after aspirin in the absence of hypothermia (Fig. 4), thereby confirming the adequacy of the aspirin treatment. Shed blood thromboxane B_2 was also markedly reduced after hypothermia in the absence of aspirin (Fig. 4). Under hypothermic conditions, aspirin resulted in a greater decrease in shed blood thromboxane B_2, but shed blood thromboxane B_2 was minimal irrespective of the presence or absence of aspirin (Fig. 4). Thus, the concentration of thromboxane generated in shed blood under hypothermic conditions in the absence of aspirin (Fig. 4) had no effect on platelet surface P-selectin or platelet aggregation in whole blood *in vitro* (Fig. 5).

DISCUSSION

In summary, aspirin does not augment hypothermia-induced platelet dysfunction in humans *in vivo*, as determined by the following 4 independent parameters in the shed blood emerging from a standardized bleeding time wound: 1) blood loss, 2) bleeding time, 3) platelet surface expression of P-selectin (reflecting α granule secretion (13)), and 4) thromboxane B_2 (a marker of platelet activation (18)).

Aspirin-induced platelet dysfunction is primarily the result of diminished generation of thromboxane A_2 because of inhibition, via irreversible acetylation, of cyclooxygenase (5). Hypothermia-induced platelet dysfunction also diminishes the generation of thromboxane A_2, via inhibition of thromboxane synthetase (1). Therefore, the present *in vivo* demonstration that aspirin does not augment hypothermia-induced platelet dysfunction is probably accounted for by the fact that thromboxane A_2 generation is already largely inhibited by hypothermia (Fig. 4). Furthermore, although aspirin may also inhibit platelet activation through a neutrophil-mediated, nitric oxide/cGMP-dependent pathway (19), hypothermia inhibits multiple aspects of platelet metabolism (4).

In this study, we demonstrate that irrespective of whether conditions are normothermic or hypothermic, aspirin has no effect on the activation-dependent increase in platelet surface P-selectin *in vivo* (Fig. 2). Therefore, the present study provides *in vivo* confirmation of previous *in vitro* studies (20,21) that demonstrate no inhibition of α granule secretion by aspirin. The lack of effect of aspirin on α granule secretion is consistent with the *in vitro* finding that α granule secretion in independent of the arachidonic acid pathway (20).

Clinical studies of hypothermic CPB have demonstrated greater post-operative blood loss in aspirinated patients compared with non-aspirinated patients (22,23). This finding is accounted for by: a) the effect of hypothermia on platelet function is rapidly reversible (4), whereas the effect of aspirin on platelet function is irreversible for the life-span of the platelet; and/or b) although the modest augmentation by aspirin of the hypothermia-induced reduction in shed blood thromboxane B_2 (Fig. 4) was insufficient to affect platelet function (Fig. 5), it may affect blood loss via less thromboxane-induced vasoconstriction.

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FIGURE LEGENDS

Fig. 1. Aspirin does not augment the hypothermia-induced prolongation of the bleeding time. The skin temperature of the forearms of normal human volunteers was equilibrated and maintained at either normothermia (32°C) or hypothermia (28°C or 22°C) before and 16 hours after the ingestion of aspirin 650 mg. Standardized Simplate II bleeding times were performed in duplicate on the forearm. Data are mean ± S.E.M., n = 14.

Fig. 2. Aspirin does not alter the hypothermia-induced inhibition of P-selectin up-regulation *in vivo*. The shed blood emerging from the bleeding time wound was fixed with 1% formaldehyde at 1 minute intervals until the bleeding stopped. The platelet surface binding of the P-selectin-specific monoclonal antibody S12 was analyzed by whole blood flow cytometry in the first and final 2 samples (the latter of which were averaged). S12 binding to peripheral blood platelets maximally activated with thrombin (5 U/mL) was assigned 100 units. Data are mean ± S.E.M., n = 7.

Fig. 3. Aspirin does not augment hypothermia-induced blood loss. Blood emerging from the bleeding time wound (shed blood) was collected onto filter paper. Blood loss was then quantitated by two methods. In panel A, after elution by cyanmethemoglobin, total shed blood hemoglobin was measured. In panel B, shed blood volume was measured by converting the opaque area on the filter paper into digitized units using a scanner and Sigmascan® digitization software. Data are mean ± S.E.M., n = 6.

Fig. 4. Effect of aspirin and hypothermia on shed blood thromboxane B_2. All blood emerging from the bleeding time wound was collected into heparin 1,000 U/mL and ibuprofen 1.9 mg/mL (to halt the production of thromboxane) and thromboxane B_2 (a stable metabolite of thromboxane A_2) was measured by radioimmunoassay. Data are mean ± S.E.M., n = 6.

Fig. 5. The concentration of thromboxane generated in shed blood under hypothermic conditions in the absence of aspirin (Fig. 4) had no effect on platelet surface P-selectin or platelet aggregation in whole blood *in vitro*. Whole blood was incubated (37°C, 5 minutes) with the indicated concentrations of the thromboxane A_2 analogue U46619. Platelet surface P-selectin was measured by whole blood flow cytometry. Platelet aggregation was measured by whole blood aggregometry. Data are mean ± S.E.M., n = 3.

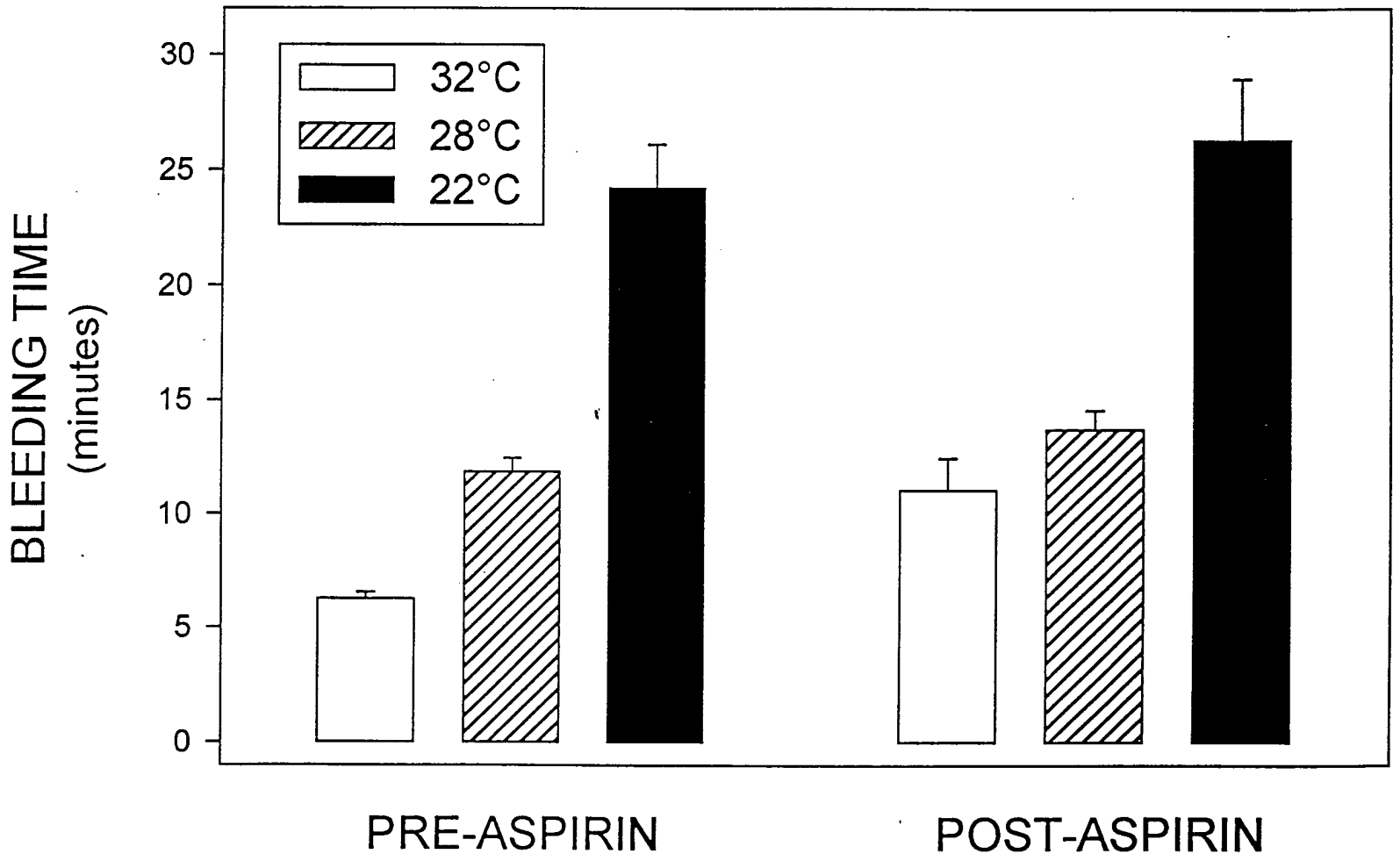
BLEEDING TIME
(minutes)
30
25
20
15
10
5
0
32°C
28°C
22°C
PRE-ASPIRIN
POST-ASPIRIN

Fig. 1

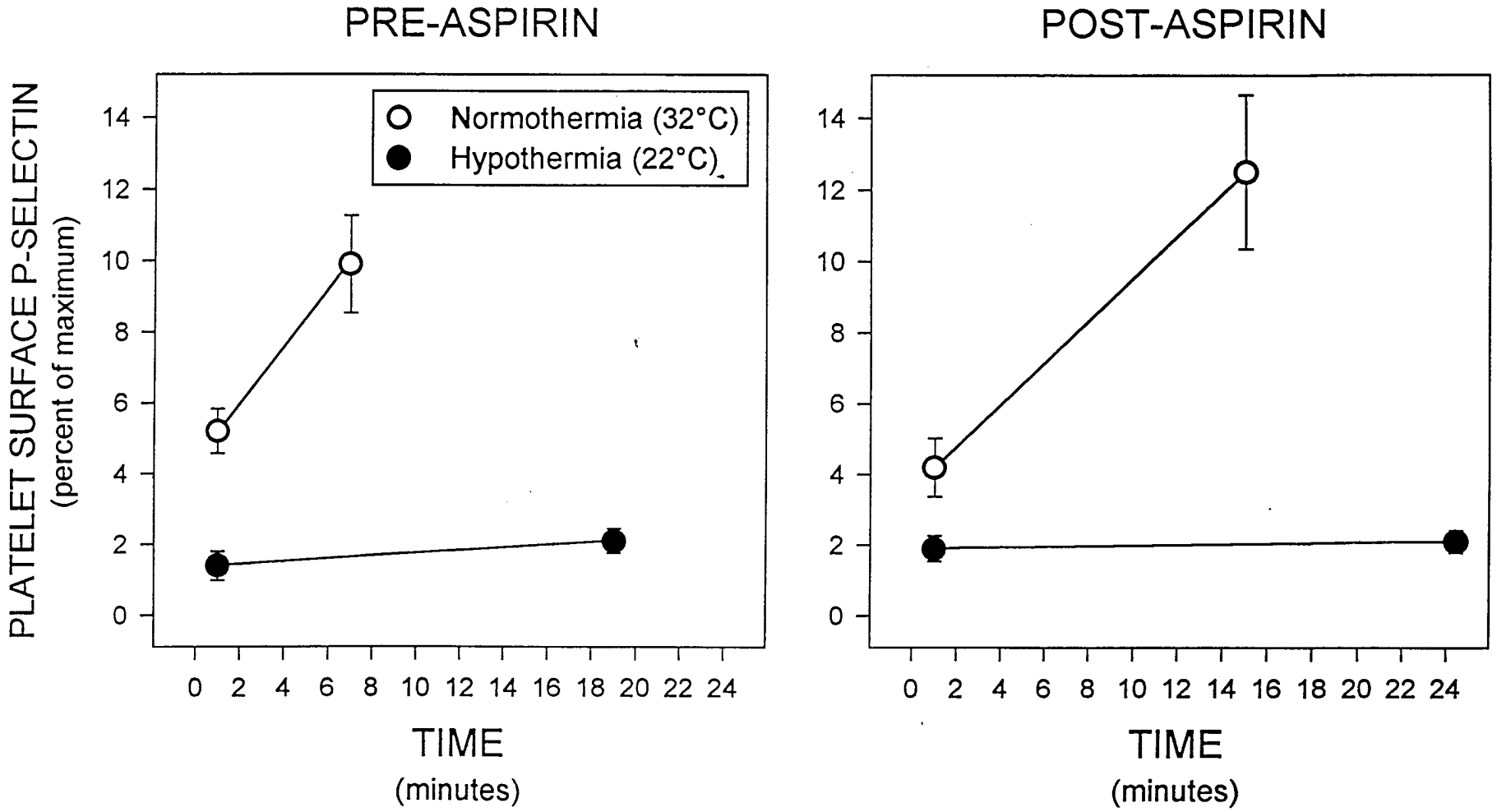
PRE-ASPIRIN
POST-ASPIRIN
PLATELET SURFACE P-SELECTIN
(percent of maximum)
Normothermia (32°C)
Hypothermia (22°C)
0
2
4
6
8
10
12
14
0 2 4 6 8 10 12 14 16 18 20 22 24
TIME
(minutes)

Fig. 2

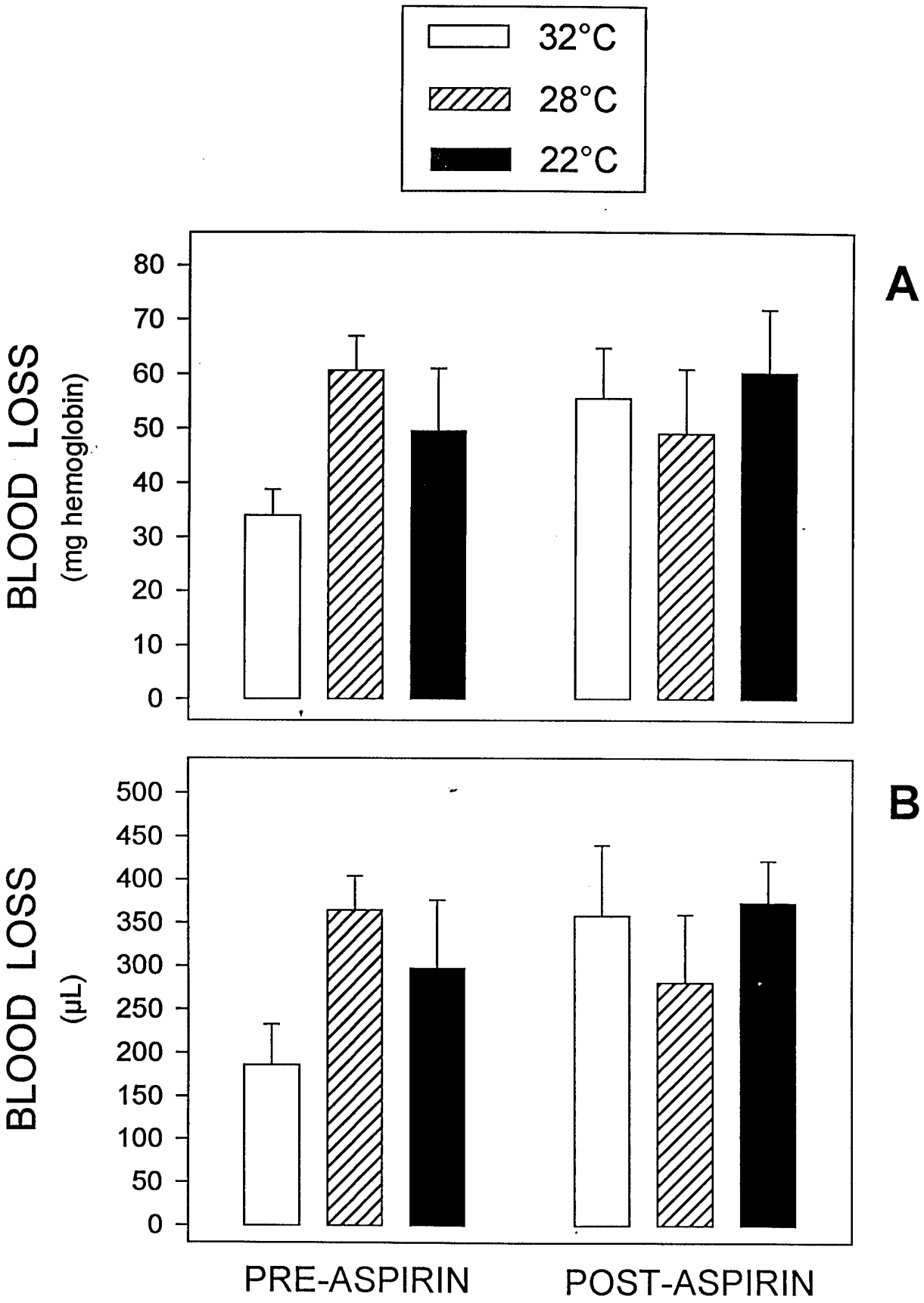
32°C
28°C
22°C
BLOOD LOSS
(mg hemoglobin)
80
70
60
50
40
30
20
10
0
A
BLOOD LOSS
(µL)
500
450
400
350
300
250
200
150
100
50
0
B
PRE-ASPIRIN
POST-ASPIRIN

Fig.3

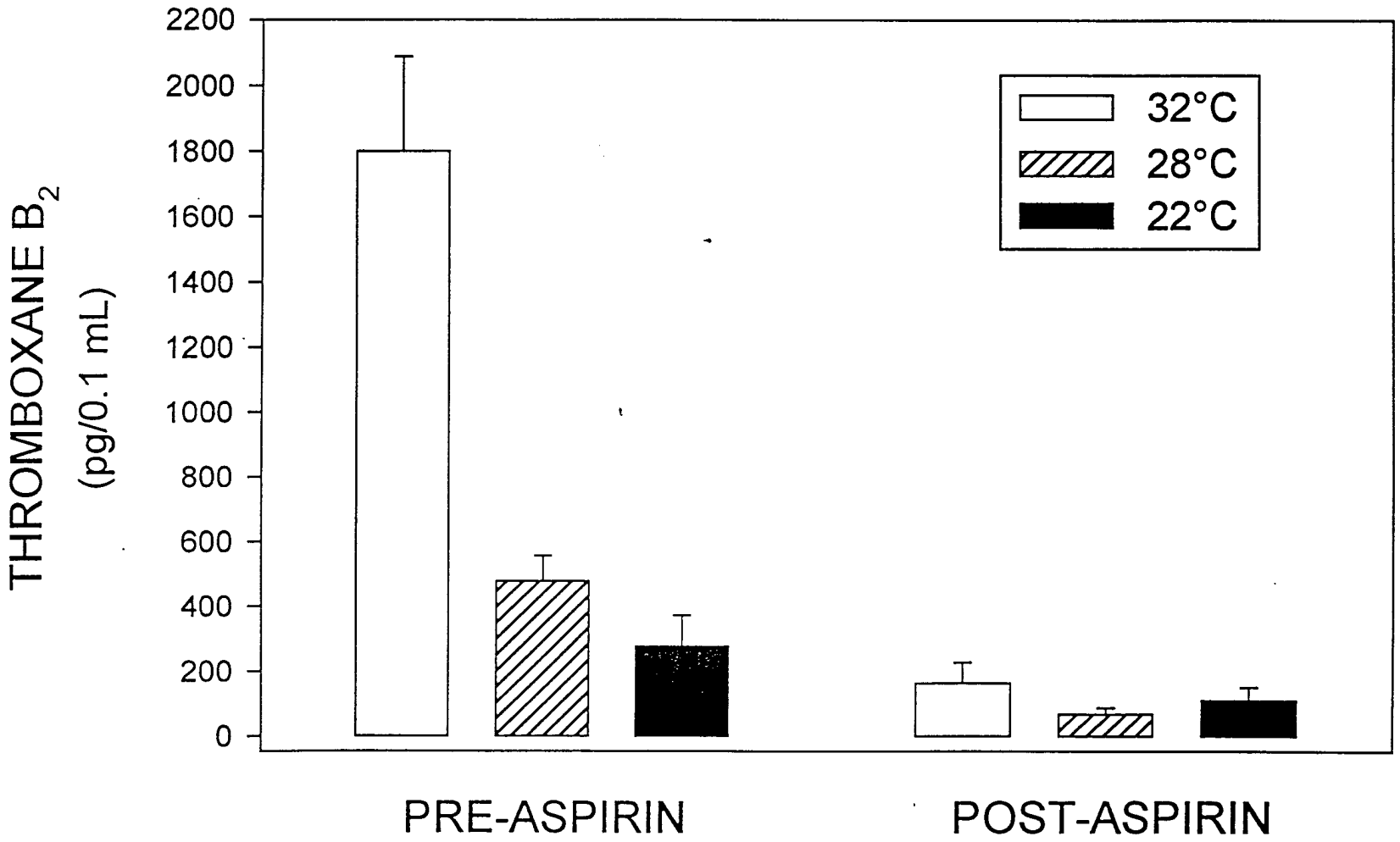
THROMBOXANE B_2
(pg/0.1 mL)
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
32°C
28°C
22°C
PRE-ASPIRIN
POST-ASPIRIN

Fig. 4

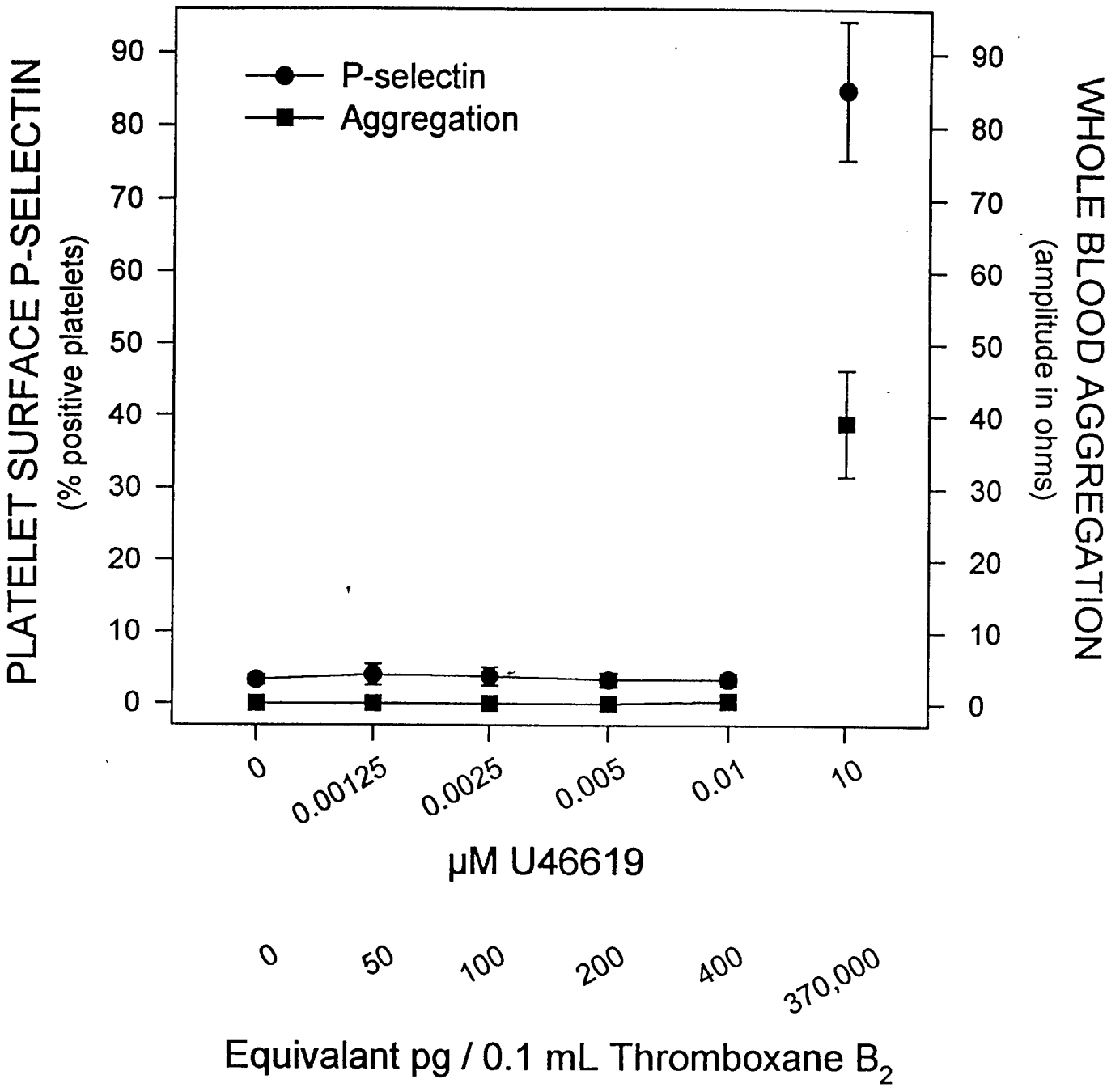
PLATELET SURFACE P-SELECTIN
(% positive platelets)
WHOLE BLOOD AGGREGATION
(amplitude in ohms)
P-selectin
Aggregation
90
80
70
60
50
40
30
20
10
0
0
0.00125
0.0025
0.005
0.01
10
µM U46619
0
50
100
200
400
370,000
Equivalant pg / 0.1 mL Thromboxane B_2

Fig. 5